FINAL REPORT
The Bottlenose Dolphin (*Tursiops truncatus*) as a Model to Understand Variation in Stress and Reproductive Hormone Measures in Relation to Sampling Matrix, Demographics, and Environmental Factors

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LONG-TERM GOALS

Our overarching goal was to develop indicators and methods to quantify chronic stress in bottlenose dolphins. Much research had focused on the stimuli which induce stress in marine mammals, as well as the hormonal mediators of the stress response. Stress may be induced by a variety of factors, including noise, pollutant or toxin exposure, presence of predators, loss of prey, human interactions, and/or habitat changes. The stress response is complex and difficult to study experimentally in marine mammals due to ethical and logistical considerations, but has been well characterized in other laboratory mammal species. In mammals, as well as other vertebrates, the stress response has two modes of operation. The fast mode involves the rapid release by the medulla of fast-acting agents, such as catecholamines, which drive the fight-or-flight response, enhancing vigilance, alertness, arousal, and attention. The catecholamines in turn play a major role in excitation of the hypothalamic-pituitary-adrenal (HPA) axis, initiating a hormonal cascade which culminates in stimulation of the adrenal cortex to secrete glucocorticoids (GCs). The delayed but more sustained response driven by GCs coordinates brain and body functions to cope with stress and facilitate recovery, adaptation, and re-establishment of homeostasis. These functions include mobilization of substrates for energy metabolism, suppression of immune and inflammatory reactions, and inhibition of bone and muscle growth. Studies of both captive and free-ranging individuals support the existence of these same stress response pathways in marine mammals.

While the HPA axis and physiological processes driven by the GCs are essential for an individual's ability to respond and adapt to stress, prolonged stimulation can overly burden the body's regulatory systems and induce deleterious effects. Prolonged elevation of GC hormones can lead to chronic immune suppression and inhibition of other energy expending hormonal systems, including disruption of reproductive function along the hypothalamo-pituitary-gonadal axis, all of which may cumulatively lead to decreased survival and/or inability to reproduce. For this reason, developing indicators and methods to quantify chronic stress in marine mammals was essential for understanding risks and long-term consequences for populations.

OBJECTIVES

Using the bottlenose dolphin as a model species, the original objectives for this project were:

- Determine correlation of hormone measures between blood and blubber.
- Develop a comprehensive understanding of factors that influence stress hormone levels and establish reference intervals, determining necessary stratifications by sex, age, and/or sampling season.
- Examine relationships among the various hormone measures, and conduct preliminary screening analysis to examine potential relationships between the stress hormones and other health measures.

Early into the project, we realized a significant need to be able to measure a broad suite of hormones from a single sample of blood or blubber, rather than having to collect and analyze multiple samples for various hormones of interest. In addition, a direct method for measurement of stress hormones is desirable that does not rely on antibody-based assays. Therefore, in 2013 we initiated a collaboration with Dr. Ashley Boggs of the National Institute of Standards and Technology (NIST) to explore direct analytical methods that would enable us to measure multiple hormones simultaneously. We therefore added an objective to the project, at no additional cost to ONR:

- Develop and validate a new method to extract and directly quantify multiple classes of hormones from a single sample.

APPROACH

The challenge of dealing with free-ranging marine mammals was reduced through selection of a species for which a broad base of biological information was already available and where appropriate samples were able to be readily obtained. We focused on common bottlenose dolphins (*Tursiops truncatus*), a species for which long-term studies of resident populations of well-known individuals were available, to study the natural variation in hormones and stress response.

Field Studies

The Chicago Zoological Society's (CZS) "natural laboratory" situation in Sarasota Bay, Florida provided unique opportunities to address questions related to stress, as a resident population of bottlenose dolphins has been studied for more than 40 years, relying upon methods which include capture-release health assessments. Stress and reproductive hormones (cortisol, aldosterone, thyroid, testosterone, progesterone) have been routinely measured in blood serum as part of the

health assessment, which also includes a complete physical examination, morphometric measurements, hearing tests, and biological sampling of skin, blubber, urine, feces, gastric contents, and blowhole. These tissue samples are analyzed for a broad suite of diagnostics. We leveraged this existing collection of data, and also conducted additional sampling during Sarasota Bay health assessments over 3 years, to help meet our proposed objectives. We also included existing data from prior (2009) capture-release health assessments along the Georgia coast.

In addition, while the logistics of capture-release sampling generally limit its utility to investigations of coastal cetaceans, remote biopsy has the potential to be a powerful tool for investigations across a range of habitats, from estuarine to nearshore and pelagic populations. To elucidate potential seasonal variation, remote biopsy samples were collected across 4 seasons in estuarine and coastal waters near Charleston, South Carolina, and across 2 seasons in the Ashepoo, Combahee and Edisto (ACE) Basin, also in South Carolina.

Laboratory Analyses
Hormone concentrations (cortisol, aldosterone, reproductive and thyroid hormones) in serum samples were analyzed by Cornell's Animal Health Diagnostic Center (AHDC) Endocrinology Laboratory. Cortisol and reproductive hormone concentrations in blubber samples matched to serum samples from the capture-release health assessments were analyzed by Dr. Kellar's (NMFS/SWFSC) laboratory using commercially available enzyme-immunoassay (EIA) kits.

In 2013, we initiated collaboration with Dr. Ashley Boggs (NIST) to develop and validate a new method using liquid chromatography tandem mass spectrometry (LC-MSMS) to directly quantify multiple classes of hormones from a single sample.

The project was a collaborative effort led by Dr. Lori Schwacke (NOAA/National Ocean Service (NOS)/National Centers for Coastal Ocean Science (NCCOS)) and Dr. Randall Wells (Chicago Zoological Society). Other collaborators and co-PIs included Eric Zolman, NOAA/NOS/NCCOS, Dr. Nicholas Kellar, NOAA/National Marine Fisheries Service (NMFS), Southwest Fisheries Science Center, Dr. Patricia Rosel, NOAA/NMFS Southeast Fisheries Science Center, Dr. Teri Rowles, NOAA/NMFS, Office of Protected Resources, Dr. Leslie Hart, NOAA/NOS/NCCOS, and Dr. Ashley Boggs, NIST.

WORK COMPLETED

Field Studies
Dolphin health assessment studies in Sarasota Bay were led by Dr. Wells. Matched blood and blubber samples were collected from dolphins in Sarasota Bay during May 2009 (n=20), May 2010 (n=10), May 2011 (n=15), and May 2012 (n=16). Additional blood samples, without blubber, were collected in July 2012 (n=10). More than 2,100 hormone measures previously obtained for Sarasota Bay dolphins were applied to this project.

Remote biopsy studies were conducted by a team that included personnel from both Dr. Schwacke and Dr. Wells' labs and were led by Mr. Zolman. Remote biopsy sampling to collect blubber samples across seasons was conducted during Fall 2011 and Winter, Spring, Summer 2012 in waters near Charleston, SC, and conducted in the ACE Basin during Winter and Summer 2012. In total, 118 blubber samples were collected (Table 1). At a minimum, 65 of the 80 dolphins sampled near Charleston were matched to the local photo-identification catalog. No comparable dolphin catalog

exists for the ACE Basin; however, one dolphin sampled there was matched to the aforementioned Charleston catalog.

Table 1. Number of biopsy samples collected near Charleston, SC and in the ACE Basin, SC.

	Fall 2011	**Winter 2012**	**Spring 2012**	**Summer 2012**
Charleston	20	20	20	20
ACE Basin		15		23
Total	20	35	20	43

Laboratory and Data Analyses

Forty-three blubber samples from previous capture-release health assessments in Sarasota and Georgia were analyzed using methods described in (Kellar et al. 2015). Although not part of this project, matched blood and blubber samples were also collected from dolphins in Barataria Bay, LA as part of the *Deepwater Horizon* (DWH) Natural Resource Damage Assessment (NRDA), and data from these NRDA studies were included in analyses to understand the effect of holding time on blubber cortisol levels, as well as to model blood-blubber relationships. Models were developed to investigate the dynamics of cortisol in different dolphin tissues and were parameterized using cortisol measurements from blubber and blood relative to handling time in live-captured individuals.

Linear mixed models were used to evaluate demographic and sampling factors contributing to observed differences in serum concentrations of adrenal hormones (i.e. cortisol, aldosterone), and relationships among these various hormones in bottlenose dolphins sampled in Sarasota Bay, Florida, USA (2000-2012). Nonparametric bootstrap methods were then used to develop stratified 90^{th} and 95^{th} percentile diagnostic reference intervals for each hormone constituent. Data from Sarasota were also combined with blood hormone measurements and ancillary health data from the NRDA studies in Barataria Bay to explore the impact of the DWH oil spill on stress hormone measures.

Methods for the homogenization of dart biopsies combined with the extraction of steroids from dolphin blubber using a QuEChERS (Quick Easy Cheap Effective Rugged Safe) extraction and two LC-MS/MS measurement methods (biphenyl separation and C18 separation methods) were developed for the quantification of baseline hormones in dart biopsies. The newly developed methods were tested on blubber from both stranded and live (remotely biopsied) dolphins. Internal standard blanks were used to calculate the limit of detection (LOD and samples were quantified using an extracted calibration curve. The biphenyl speparation and C18 separation methods were both explored for the utility in the measurement of cortisol, as well as for the measurement of the other steroids of interest related to stress response and/or reproduction.

RESULTS

A number of important findings have resulted from this investigation. The two most important findings relate to: 1) the influence of sampling variables and the potential for effects from chemical pollutants on cetacean stress response, and 2) the surprising correlation of cortisol concentrations in blood and blubber following stimulation, with blubber concentrations exibiting a time lag in relation to blood concentrations. In addition, we have established a new capability for measuring a broad suite of steriod hormones in blubber that can be used for direct quantification of both stress and reproductive measures from a single small remote dart biopsy sample.

Influence of Sampling Variables and Effect of Chemical Pollutants on Stress Response

The analysis of adrenal hormone (cortisol, aldosterone) data from Sarasota Bay (2000-2012) found that serum cortisol concentrations were significantly associated with elapsed time from initial stimulation (i.e., time the the net was set around the dolphins) to sample collection, but that other demographic variables (age, sex, time of day) were not influential (Hart et al. 2015). Reference intervals were therefore constructed for serum cortisol based on elapsed sampling time (Hart et al. 2015). The reference intervals are important for identification of "abnormal cases" to interpretate of adrenal hormone measures across cetacean populations exposed to varying types and degrees of stressors. These data and analyses were applied to assess the stress response and associated health data from dolphins sampled from Barataria Bay, LA, an area heavily oiled following the *Deepwater Horizon* oil spill. Based on the findings, it was determined that Barataria Bay dolphins demonstrated evidence of hypoadrenocorticism, consistent with adrenal toxicity as previously reported for laboratory mammals exposed to oil (Schwacke et al. 2014). These results demonstrate the critical need to understand exposure to multiple stressors and potential synergistic, additive, or even antagonistic effects that may occur among exposures (e.g., oil exposure and acoustic disturbance) that are known to affect marine mammal stress responses.

Dynamics of Cortisol in Blood and Blubber Relative to Handling Time in Stimulated (Live-captured) Cetaceans

Our model of blubber and blood cortisol concentrations considering elapsed time from initial capture was the first to suggest that blubber cortisol concentration increases with handling time following stimulation (in this case, stimulation from capture and restraint). Using data from Sarasota Bay capture-release efforts, a positive correlation of blubber cortisol concentration and handling time following encirclement with a net was established (Figure 1). Adding additional samples from dolphin live capture projects in Georgia and Louisiana, our analysis demonstrated blubber cortisol levels correlate with cortisol level in blood, but with a time lag. A series of models applied to matched samples of blood and blubber found that the increase in blubber cortisol following stimulation lags behind the increase in serum cortisol, taking around 1.5 to 3 hours to reach a plateau of around 7 ng/g (Figure 2).

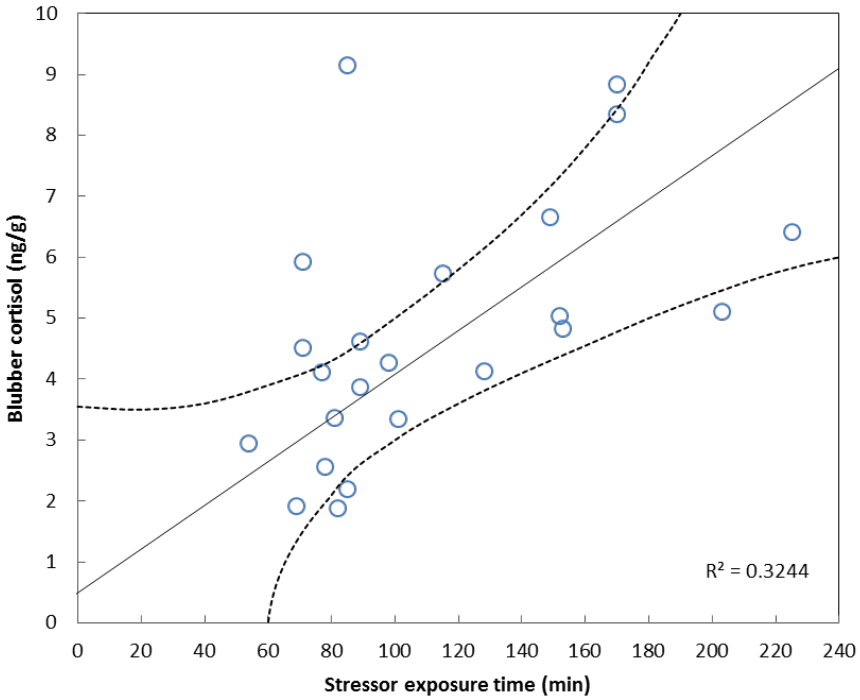

Figure 1. Cortisol measured in blubber samples vs. time from net encirclement for bottlenose dolphins.

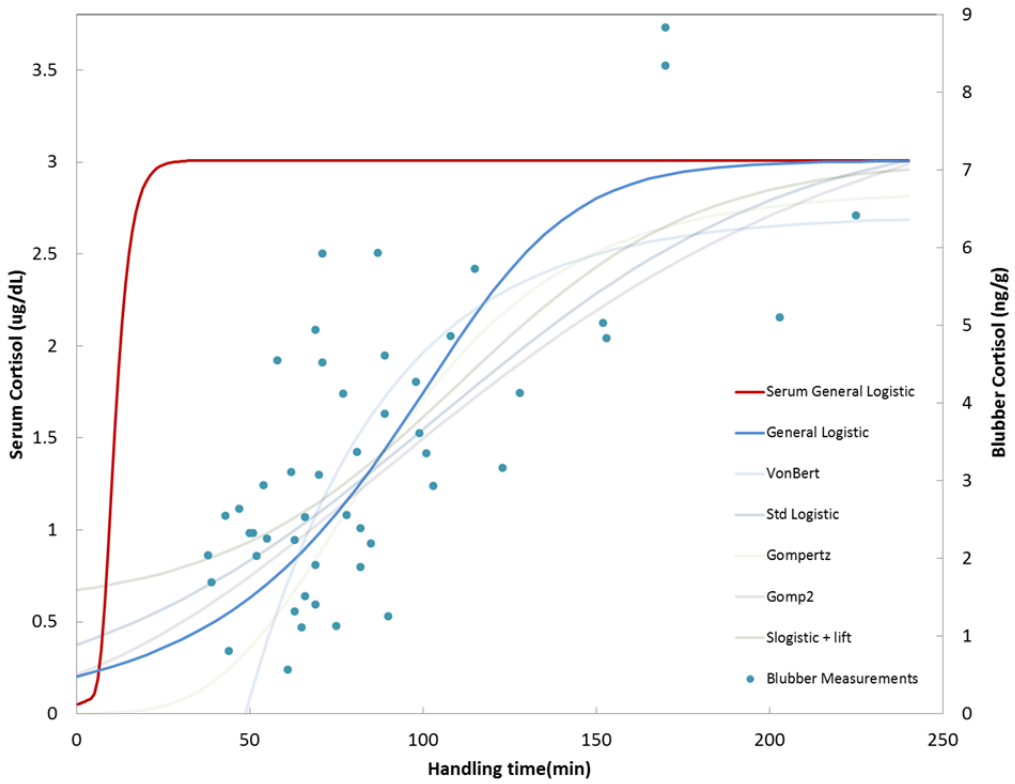

Figure 2. Models for cortisol dynamics in blood and blubber. Handling time is the time from when a net was deployed around the dolphins.

New Capability for Direct Measurement of a Broad Suite of Steroid Hormones

Our research developed methods that allow for the direct quantification of a broad suite of steriod hormones. We determined that a homogenization technique involving mincing dart biopsies on dry ice followed by bead homogenization, a QuEChERS extraction, and two LC chromatography methods (biphenyl separation and C18) followed by MS/MS detection allows for the quantification of low concentration baseline (*i.e.*, unstimulated) steroid hormones in relatively small dart biopsy samples using an extracted calibration curve and isotope dilution.

Chromatography including separation and peak shape for the progestogens and androgens were excellent using the biphenyl separation method (Figure 3), but cortisol and cortisone displayed background interference and poor peak resolution. Conversely, cortisol and cortisone peak shape and detection were excellent using the C18 separation method (Figure 4), but the remaining steroids eluted very close together (within 2 minutes) which could cause inaccuracies in quantification due to the similarity in compound mass and structure shared by the steroid hormones. Concentrations of the various steriods using the two methods were compared using blubber from a dolphin that live stranded and was then euthanized (Table 2). We established that the two LC methods in combination can be used to simultaneously measure a broad suite of hormones that are of interest for understanding stress response and potential relationships with reproductive status.

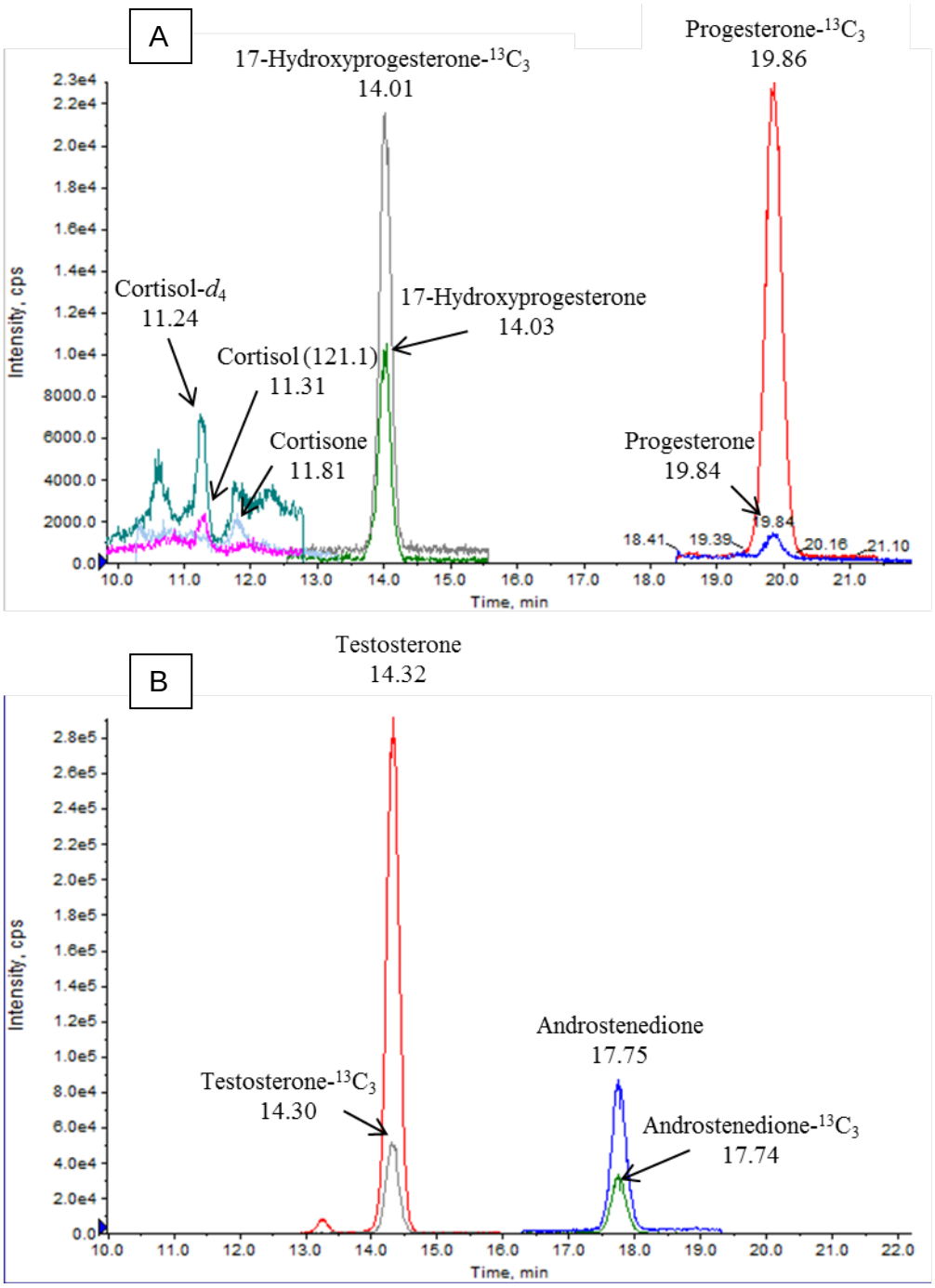

Figure 3. Representative chromatograms and retention times of steroids measured in this study from dart biopsied dolphin blubber (sample ID TYP-140730-01) using the biphenyl separation method. Panels are split for peak clarity. A) Corticosteroids and progestogens. B) Androgens.

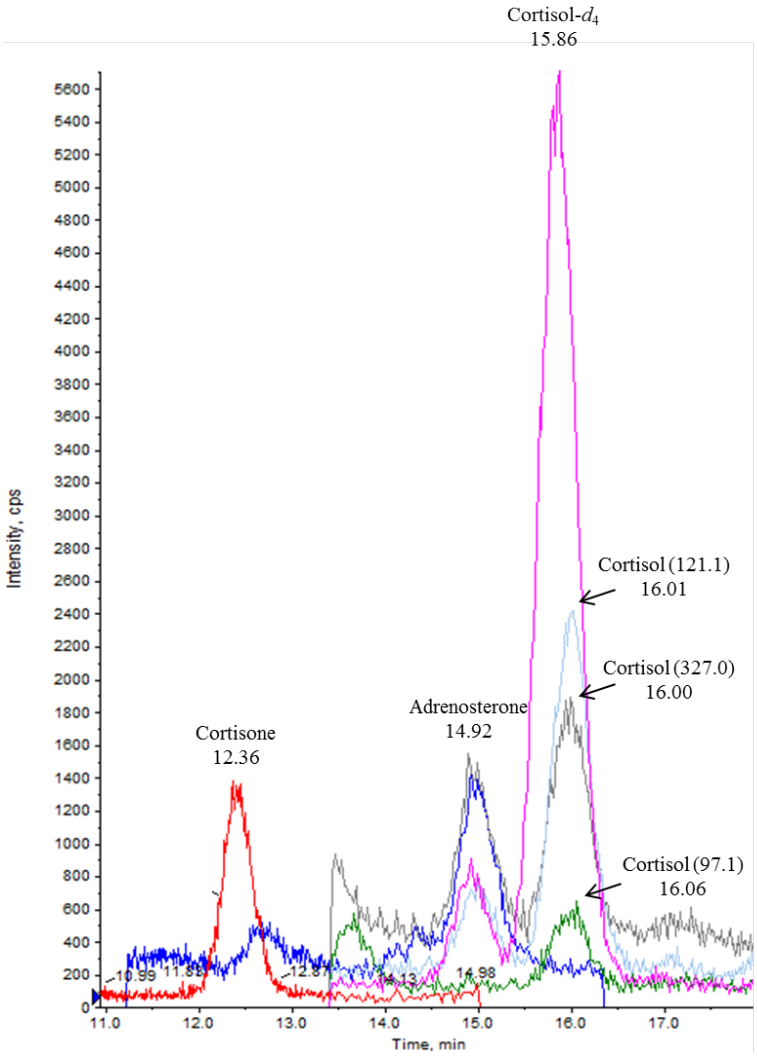

Figure 4. Representative chromatograms and retention times of steroids measured in this study from dart biopsied dolphin blubber (sample ID TYP-140730-01) using the C18 separation method.

Table 2. Summary statistics for concentrations (ng/g) of steriods measured using two methods for LC-MS/MS for SC1426, a dolphin that live stranded in South Carolina and was then euthanized.

	Biphenyl			C18			
Analyte	Mean (ng/g)	Stdev	RSD (%)	Mean (ng/g)	Stdev	RSD (%)	Percent difference
11-deoxycorticosterone*	< LOD			1.35	0.10	7.61	
11-deoxycortisol	0.924	0.134	14.5	0.621	0.103	16.6	39.2 %
17-OH-Progesterone	1.42	0.14	10.2	1.38	0.25	18.4	2.86 %
Adrenosterone	< LOD			0.476	0.085	17.8	
Androstenedione	2.00	0.23	11.5	1.96	0.22	11.2	2.02 %
Corticosterone	1.40	0.16	11.5	1.25	0.06	4.98	11.3 %
Cortisol (121.1)	14.1	2.0	14.4	10.2	0.6	5.84	32.1 %
Cortisol (97.1)	25.0	8.8	35.1	9.94	0.22	2.26	86.2 %
Cortisol (327.0)	NA			9.75	0.48	4.94	
Cortisone	9.08	0.41	4.6	5.82	0.10	1.67	43.8 %
Dehydroepiandrosterone	ND			20.2	8.8	43.5	
Dihydrotestosterone	< LOD			0.0169	0.0641	380	
Pregnenolone	ND			NA			
Progesterone	1.96	0.20	9.9	1.82	0.32	17.9	7.41 %
Testosterone	2.74	0.16	5.8	2.31	0.94	40.8	17.0 %

Notes: * = Not included in calibration curve therefore quantification is inaccurate but RSD remains informative; < LOD = Less than the limit of detection; ND = Not detected; NA = Not analyzed; Green boxes and text represent an RSD of under 15 %; Yellow boxes and text represent an RSD under 20 %. Purple cells represent compounds with good agreement which is a percent difference of less than 20 % for the two separation methods.

Blubber samples from stranded dolphins demonstrate much higher concentrations of stress hormones when compared to samples that are collected quickly prior to the dolphin having time to effectively respond to sampling stress [e.g., bycatch or remote biopsy see (Kellar et al. 2015)]. However, we were still able to detect steroid concentrations in remote biopsy samples using both LC/MS-MS methods (Tables 3 and 4). We determined noticeable differences in detection according to the sex of the animals sampled. Using the biphenyl method on two representative male samples, 17-hydroxyprogesterone, androstenedione, cortisol, and testosterone were all quantifiable. Using the biphenyl method on two representative female samples, only cortisol and sometimes corticosterone were quantifiable. Using the C18 method on the male samples, androstenedione and cortisol were quantified. In the female sample, only cortisol was quantifiable.

Table 3. Concentrations of steroids in dart biopsied dolphin blubber (ng/g) measured using the biphenyl method.

Analyte	LOD	TYP140227-01 (Female)	TYP140730-01 (Male)	TYP140801-02 (Male)	TYP140806-03 (Female)
11-Deoxycortisol	0.181	< LOD	0.200	< LOD	< LOD
17-OH-Progesterone	0.058	< LOD	3.155	1.252	< LOD
Adrenosterone	0.231	< LOD	< LOD	0.121	< LOD
Androstenedione	0.067	< LOD	15.223	4.330	< LOD
Corticosterone	0.128	0.690	< LOD	< LOD	< LOD
Cortisol (121.1)	0.357	0.694	1.215	0.800	0.392
Cortisol (97.1)	0.480	0.086	0.682	3.174	1.554
Cortisone	0.339	< LOD	0.781	0.438	< LOD
Dehydroepiandrosterone	0.477	< LOD	< LOD	< LOD	< LOD
Dihydrotestosterone	1.442	< LOD	< LOD	< LOD	< LOD
Pregnenolone	1.222	< LOD	< LOD	< LOD	< LOD
Progesterone	0.091	< LOD	0.473	0.262	0.150
Testosterone	0.074	< LOD	31.529	10.110	< LOD

Notes: < LOD = Less than the limit of detection; NQ = Not quantified; Blue text = below the lowest calibration standard; Grey cell = less than 15 % RSD; Blue cell = less than 20 % RSD.

Table 4. Concentrations of steroids in dart biopsied dolphin blubber (ng/g) measured using the C18 method.

Analyte	LOD	TYP140227-01 (Female)	TYP140730-01 (Male)	TYP140801-02 (Male)	TYP140806-03 (Female)
11-Deoxycortisol	0.074	< LOD	0.084	< LOD	< LOD
17-OH-Progesterone	0.221	< LOD	2.594	1.314	< LOD
Adrenosterone	0.090	< LOD	0.149	0.108	< LOD
Androstenedione	0.216	< LOD	18.300	5.297	< LOD
Corticosterone	0.050	ND	ND	ND	ND
Cortisone	0.140	0.160	0.483	0.217	0.214
Cortisol (121.1)	0.263	0.528	1.081	0.763	0.803
Cortisol (97.1)	0.207	0.539	0.985	0.767	0.736
Cortisol (327.0)	0.323	0.543	1.149	0.805	0.926
Dehydroepiandrosterone	7.935	< LOD	< LOD	< LOD	< LOD
Dihydrotestosterone	0.003	< LOD	4.428	0.824	0.013
Progesterone	0.229	< LOD	0.528	0.512	0.337
Testosterone	0.523	< LOD	50.844	11.719	< LOD

Notes: < LOD = Less than the limit of detection; ND = Not detected; NQ = Not quantified; Blue text = below the lowest calibration standard; Red text = above the highest calibration standard; Grey cell = less than 15 % RSD; Blue cell = less than 20 % RSD.

Based on these results we conclude that 17-hydroxyprogesterone, testosterone, androstenedione, and cortisol can be readily quantified in male samples with a high likelihood of detecting cortisone and progesterone after improvement of the calibration curve. In female samples, cortisol and elevated progesterone concentrations indicating pregnancy can be quantified with a high likelihood of quantifying cortisone and baseline progesterone after improvement of the calibration curve. This demonstrates that the two LC methods in combination can provide a simultaneous measurement of a broad suite of hormones that are of interest for understanding stress response and reproductive status.

IMPACT/APPLICATIONS

Our results to date provide tremendous new insight into the dynamics of cortisol in both blood and blubber for dolphins experiencing acute stress, as well as provide critical baseline information on cortisol and other hormone concentrations in wild dolphin populations. This understanding is absolutely essential for future stress studies in dolphins and other cetaceans in order to appropriately interpret stress hormone measures. Furthermore, the reference intervals produced by this study provide the basis for evaluation of animal health that is already being used for population assessments to understand the potential adrenal toxicity of chemical stressors.

The analytical methods developed through this study can be used for the direct quantification of a broad suite of steroid hormones from a single small remote biopsy sample. The methods will enable studies to explore relationships and map trends in stress and reproductive hormones, and with less stress to the animal, less sampling effort and lower costs than sampling associated with capture-release. This will be a very important tool for initial evaluation of dolphin populations to help determine if full-scale capture-release health assessments might be warranted for further investigation of populations of concern. The methods will greatly expand capabilities for the scientific community to conduct studies to evaluate stress response and potential associated reproductive effects in a broad range of cetacean species and stocks.

TRANSITIONS

The reference intervals for body mass index and serum cortisol concentrations following capture established and published by this study (Hart et al. 2013, Hart et al. 2015) are currently being used by NOAA and others to evaluate disease cases as part of bottlenose dolphin population health assessments and for dolphins under human care at a number of facilities.

RELATED PROJECTS

None.

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